

## CD23 defines two distinct subsets of immature B cells which differ in their responses to T cell help signals

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### Abstract

Transitional immature B cells undergo apoptosis and fail to proliferate in response to BCR cross-linking, thus representing a target for negative selection of potentially autoreactive B cells *in vivo*. In agreement with recent reports, transitional B cells were divided into developmentally contiguous subsets based on their surface expression of CD23. When transferred, CD23<sup>+</sup> transitional B cells readily localized to the splenic follicles and the outer PALS. Compared with CD23<sup>-</sup> transitional B cells, CD23<sup>+</sup> transitional B cells proliferated more vigorously and were rescued from BCR-induced apoptosis to a greater degree, by T cell help signals. However, both CD23<sup>-</sup> and CD23<sup>+</sup> transitional B cells failed to up-regulate CD86 (B7-2) in response to BCR ligation. These findings demonstrate that phenotypically defined subsets within the transitional B cell population are functionally distinct. Specifically, responsiveness to T cell help is a late acquisition corresponding to the stage when the B cells gain access to peripheral compartments enriched in antigen and activated T cells. The failure of transitional B cells to up-regulate CD86 to BCR-mediated stimulation suggests a unique interaction between transitional B cells and T cells with implications for tolerance in the T cell compartment.

### Introduction

B cell development proceeds in an orderly and sequential manner giving rise to mature cells capable of interacting with antigen through surface Ig (1-4). Early B cell development in the bone marrow (BM) has been well characterized with the development of several schemes to classify subsets within the pro-B and pre-B cell populations (1,2). The final developmental stage in the BM before emigration is the late immature B cell that has high expression of surface IgM (slgM). These cells leave the BM and further develop into mature cells. Of the  $\sim 2 \times 10^7$  IgM<sup>+</sup> B cells which develop in the murine BM daily, only  $\sim 10\%$  exit to the periphery. Of these émigrés, a larger proportion progresses to the immature B cell pool (5,6). In contrast to our knowledge of early events in the BM, the features of later peripheral development are relatively obscure. Specifically, the factors that determine the selection of late immature B cells into the long-lived pool are not well understood despite their potential importance in immune repertoire formation.

Transitional immature B cells lie developmentally between BM immature and peripheral mature B cells. They represent recent BM émigrés and have phenotypic characteristics distinct from mature B cells (5,7,8). As with late stage BM immature B cells, they are heat-stable antigen (HSA)<sup>high</sup> (CD24) and slgM<sup>high</sup>. In a manner similar to HSA<sup>high</sup>/slgM<sup>high</sup> BM immature B cells, they respond to *in vitro* antigen receptor cross-linking by apoptosis instead of proliferation (9,10). Transitional B cells represent an important target for negative selection to self antigens *in vivo* and likely play an important role in maintaining tolerance to peripheral antigens not present in the BM.

We have previously demonstrated the ability of activating anti-CD40 and soluble IL-4 to block the apoptotic response of transitional B cells to BCR cross-linking and change it to an activation response (11). The ability to redirect the BCR-induced negative selection response by signals

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associated with activated T cells implies that a mechanism exists to potentially recruit antigen-reactive immature B cells into an ongoing immune response. For this reason, we considered that a component of the transition from immature to mature B cells would be the ability to respond to T cell help signals and redirect the intrinsic apoptotic response towards a BCR-induced activation response.

Transitional B cells themselves represent a heterogeneous population of cells with phenotypic variability which can be further subdivided by the presence or absence of surface proteins such as CD21, CD23 and IgD (5,12). In agreement with previous reports, we found that HSA<sup>int</sup> transitional B cells could be divided into two subsets defined by their expression of CD23, and extended our observations to examine the functional characteristics of the two subtypes. As was implied by previous studies (11), neither subset differed detectably in their intrinsic response to BCR cross-linking. However, functional differences were found between these two populations in their relative abilities to respond to T cell help.

## Methods

### Animals

Female BALB/c mice were bred in the animal facility of the University of Pennsylvania and used at 8–12 weeks of age for all experiments. Transitional B cells were harvested and purified from the spleens of mice 13 or 14 days after irradiation with 500 rad (5,9). These auto-reconstituted B cells are >98% HSA<sup>high</sup>. For some experiments, auto-reconstituted splenocytes were harvested 12–45 days after irradiation.

### Antibodies and reagents used in B cell stimulation and FACS analysis

Rabbit anti-IgM F(ab)<sub>2</sub> fragments were prepared from IgG collected from rabbits immunized with an IgM<sup>a</sup> antibody as previously described (13). IL-4 supernatant was prepared from X4 cells provided by Dr Fritz Melchers and anti-CD40 (1C10) was generously provided by Dr Maureen Howard. The following antibodies were used for FACS analysis: anti-CD86 (GL-1), anti-CD21 (7G6), anti-CD22 (Cy34.1), anti-CD23 (E3B4), anti-IgM<sup>a</sup> (AMS 9.1), anti-IgM<sup>d</sup> (DS-1) and anti-B220 (RA3-6B2), and were purchased from PharMingen (San Diego, CA).

### Cell preparation

Spleens from non-irradiated or post-irradiated mice were dissected and cells were dispersed with the frosted surface of glass slides. The cell suspension was collected and treated with anti-Thy-1.2, rabbit complement and DNase I for 1 h. The small population of immature B cells was depleted from unirradiated adult spleens with the addition of 493 antibodies, a recently isolated mAb specific for immature B cells (6), followed by MAR 18.5 during the complement lysis step. MAR18.5 is an anti-rat-k antibody which enhances cytolytic by non-complement fixing antibodies. After red blood cell lysis in Gey's solution, the cells were centrifuged over a 50%/75% Percoll gradient (Pharmacia Biotech, Piscataway, NJ). Cells separating at the 50/75% interface were collected for

use in the experiments. Typical preparations were 90–95% pure for B cells

BM B cells were prepared by separating and crushing the femurs and tibias of mice with a mortar and pestle, and passing the suspension over nylon wool to remove bone. The cells were treated to remove contaminating non-B cells and pro-B cells by incubation in an antibody mixture and rabbit complement. The following antibodies were used to eliminate the respective cell types: HC-13-4 (T cells), F4/80 and Mac-1 (macrophages), RB6-8C5 (granulocytes), S7 (pro-B cells) and M175 (erythrocytes). MAR18.5 was also added.

For some experiments, CD23<sup>+</sup> and CD23<sup>-</sup> transitional cells were further purified and separated by cell sorting on a FACSort device (Becton Dickinson, Mountain View, CA) in the Flow Cytometry Facility of the Cancer Center at the University of Pennsylvania after staining with anti-CD23 and anti-B220. For cell transfer experiments, the purified B cells were stained only with CD23 and sorted according to CD23 status. Staining and separation were performed at 4°C.

### Thymidine incorporation

B cells were cultured in B cell assay media containing RPMI 1640, supplemented with 10% heat-inactivated FCS (Defined; Hyclone, Logan, UT), 2 mM L-glutamine, non-essential amino acids, 100 µg/ml penicillin and 5×10<sup>-5</sup> M 2-mercaptoethanol at 37°C in 6% CO<sub>2</sub>. Cells were plated at a final density of 1×10<sup>6</sup>/ml in flat-bottomed 96-well plates (Falcon) in 200 µl of assay media alone or containing the specified reagents. [<sup>3</sup>H]thymidine (50 µCi/well) was added at 48 h and the plates were harvested 16 h later in a PhD harvester using Whatman filters. Radioactivity was counted in an LKB scintillation counter. Means ± SEM were determined for each triplicate.

### Apoptosis assay

Cells (1–2×10<sup>6</sup> as indicated) were cultured in 200 µl of assay media in sterile snap-top tubes with the indicated reagents. Cells were harvested at 18 h by one wash with PBS containing 2% FCS and 0.02% azide followed by overnight incubation at -20°C in 70% EtOH/PBS. The following day, cells were washed in PBS, resuspended in PBS containing 0.1% azide, 10 µg/ml propidium iodide (Sigma) and 50 µg/ml RNase A, and incubated at room temperature for 2–16 h. Triplicate samples were then analyzed on a Becton Dickinson FACSCalibur using CellQuest software. The percent apoptotic cells as represented by <2n DNA content was calculated by cell cycle analysis.

### Carboxyfluorescein succinimidyl ester (CFSE) labeling of B cells and transfer into adult BALB/c mice

After purification and sorting, B cells were resuspended at 5×10<sup>6</sup>/ml of PBS which was kept at room temperature. To these cells, equal volume of 5 µM CFSE in PBS (room temperature) was added for 3 min. The reaction was quenched with one-fifth volume cold FBS and then washed in PBS. Between 5 and 10×10<sup>6</sup> CFSE-labeled B cells were injected into tail veins of adult BALB/c recipient mice in 500 µl of PBS.

#### Immunohistochemistry

At 16 h after i.v. transfer of CFSE-labeled B cells, the recipient mice were sacrificed, and the spleens and lymph nodes were harvested. A part of each spleen was taken for preparation into a cell suspension and analyzed by flow cytometry. The remainder of the spleens were prepared and stained as previously described (14,15). The following antibodies were used: anti-CD4-biotin (GK 1.5 grown as supernatant), anti-CD22-biotin (Cy34.1; PharMingen) and anti-FITC-alkaline phosphatase (Sigma) to detect CFSE-labeled cells. The biotin-conjugated reagents were detected with the secondary reagent streptavidin-horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL). Alkaline phosphatase and horseradish peroxidase were developed with the substrate Fast-Blue BB base (blue) and 3-amino-9-ethylcarbazole (red) respectively.

#### Results

##### *Transitional B cells can be subdivided into CD23<sup>+</sup> and CD23<sup>-</sup> populations*

Transitional B cells comprise 5–15% of the adult splenic B cell population and can be distinguished by HSA<sup>high</sup> staining (7). Although these cells have traditionally been regarded as a homogeneous population, recent reports have delineated subsets according to surface marker differences. We found that HSA<sup>high</sup> B cells could be divided most readily into two distinct subsets based upon their expression of CD23 (Fig. 1A). The CD23<sup>+</sup> B cells are comprised mostly of CD21<sup>+</sup> immature B cells, with the remainder made up by marginal zone B cells expressing high levels of CD21 (Fig. 1A).

While the bone marrow of adult BALB/c mice contained almost exclusively CD23<sup>+</sup> B cell precursors, the spleens of the same animals contained predominantly CD23<sup>-</sup> B cells. The spleens from auto-reconstituting mice contained two subpopulations with roughly equal numbers of CD23<sup>+</sup> and CD23<sup>-</sup> B cells 14 days after sublethal irradiation (Fig. 1B and C). Thus, contrary to traditional views, and in agreement with recent reports (12), CD23 status did not exclusively mark the mature B cells, but characterized a significant subset of the transitional B cells.

We examined the BM of auto-reconstituting mice and found no increase in CD23<sup>+</sup> B cells compared to the BM of unirradiated mice, confirming that the sublethal irradiation process did not artificially lead to the up-regulation of CD23 in developing B cells (data not shown).

##### *CD23 status represents a continuum in the maturation process*

In addition to CD23, we found that other B cell surface proteins, including IgD, CD21, CD22 and B220, are regulated during development from BM immature B cells to peripheral mature B cells. The expression of these surface proteins on transitional B cells was compared to that of unirradiated adult splenic B cells with respect to their CD23 status. Auto-reconstituted transitional B cells were stained with antibodies for CD23 as well as for the B cell markers CD21, CD22, IgD, B220 and HSA. There was a progressive increase in surface expression levels of CD21, CD22 and B220 from CD23<sup>+</sup> to CD23<sup>-</sup> transitional B cells and then to mature B cells. While

CD23<sup>-</sup> transitional B cells expressed low levels of IgD, CD23<sup>+</sup> transitional B cells and B cells from unirradiated adult spleens expressed similarly higher levels. Lastly, HSA levels were found to be comparable, although slightly higher, in the CD23<sup>+</sup> transitional B cells compared to CD23<sup>-</sup> transitional B cells, while they were significantly lower, as expected, in the B cells from unirradiated adult spleens (Fig. 2).

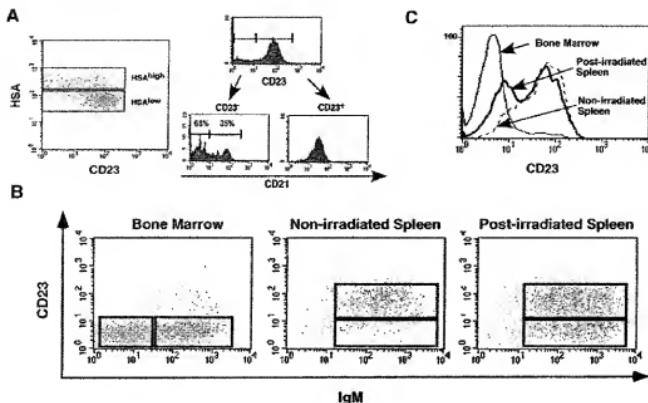
As the surface expression of these proteins increased as the B cells matured, it was probable that the CD23<sup>+</sup> transitional B cell subset was a precursor to the CD23<sup>-</sup> transitional B cell subset. This is supported by previous data demonstrating that sorted IgD<sup>+</sup> or CD23<sup>+</sup> cells injected into a mouse developed into CD23<sup>+</sup> cells (5,7). We explored this further by determining the phenotypic and functional properties of B cells from auto-reconstituting spleens over a range of time following sublethal irradiation. In the BM, all IgM<sup>+</sup> B cells were CD23<sup>+</sup> and HSA<sup>high</sup> (Fig. 3A and B). Thirteen days after sublethal irradiation, auto-reconstituting B cells were evenly distributed between CD23<sup>+</sup> and CD23<sup>-</sup> HSA<sup>high</sup> cells. Two days later, the B cells were still homogeneously HSA<sup>high</sup> but the ratio of CD23<sup>+</sup> to CD23<sup>-</sup> cells had increased. HSA<sup>low</sup> cells comprised the majority of B220<sup>+</sup> cells by day 19 and by day 22 they were nearly at levels observed in unirradiated splenic B cells. The alteration of phenotypes was consistent with a peripheral B cell developmental pathway progressing from CD23<sup>+</sup>/HSA<sup>high</sup> cell (early transitional) to CD23<sup>-</sup>/HSA<sup>low</sup> cell (late transitional) and then finally to CD23<sup>-</sup>/HSA<sup>low</sup> cells (mature). These data correspond to the T1 and T2 transitional B cell subsets described by Loder *et al.* (12), and are consistent with previous reports showing that transitional B cells require 3–4 days to develop into mature B cells *in vivo* (5,7).

##### *CD23<sup>+</sup> and CD23<sup>-</sup> transitional B cells respond similarly to BCR cross-linking*

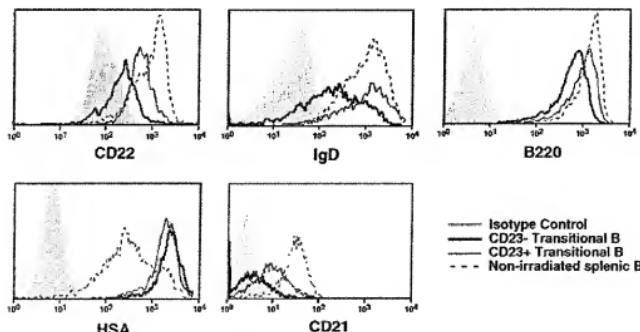
Transitional B cells isolated from auto-reconstituted spleens undergo apoptosis in the presence of BCR cross-linking, whereas mature B cells respond to the same stimulus by proliferation (11). We found that CD23<sup>+</sup> and CD23<sup>-</sup> transitional B cells shared these critical functional characteristics. In contrast to mature B cells, CD23<sup>+</sup> and CD23<sup>-</sup> transitional B cells are both susceptible to apoptosis in response to BCR cross-linking. CD23<sup>+</sup> transitional B cells exhibited a higher spontaneous ligand-independent apoptosis rate as well as a slightly higher level of apoptosis for any given amount of BCR stimulation compared to their CD23<sup>-</sup> counterparts (Fig. 4A). In addition, neither CD23<sup>+</sup> nor CD23<sup>-</sup> immature B cells proliferated to BCR cross-linking, whereas both were induced to proliferate to lipopolysaccharide (LPS) (Fig. 4B). The different degrees of proliferation to LPS in the two subsets may be related to survival differences, but the exact mechanism remains unclear.

##### *CD23<sup>+</sup> transitional B cells localize to the B cell follicles and the outer PALS*

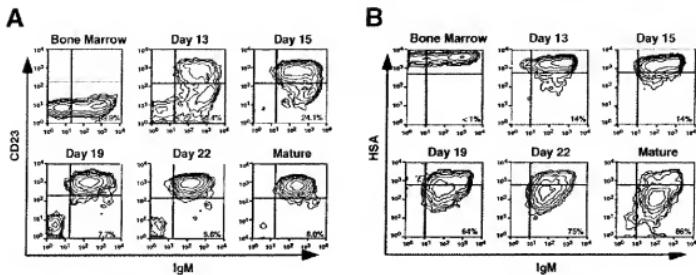
Recirculating mature naïve B cells enter the spleen via the marginal zone sinuses (16). If these virgin B cells encounter antigen, they stop their recirculation in the T cell zone (17,18) and undergo a blastogenic response, forming primary (T zone) B blasts (15,19). Some go on to differentiate into short-lived plasma cells, whereas others continue onto primary



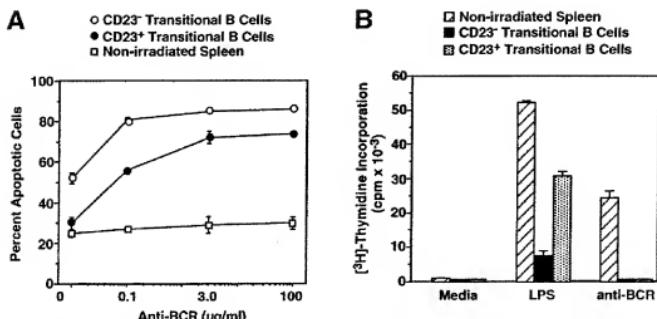
**Fig. 1.** Presence of CD23<sup>-</sup> and CD23<sup>+</sup> HSA<sup>high</sup> transitional immature B cells in spleens of non-irradiated adult and day 13–14 post-irradiated mice. (A) Purified B splenocytes from non-irradiated mice. The left figure shows the two subpopulations of HSA<sup>high</sup> B cells, whereas the right figure demonstrates that the CD23<sup>-</sup> B cells are comprised of both immature (CD21<sup>-</sup>) and marginal zone (CD21<sup>+</sup>) B cells. The cells are gated on B220<sup>+</sup> cells. (B) CD23 status of purified B cells from bone marrow and spleens of non-irradiated mice, and from purified B splenocytes of post-irradiated mice. The cells were gated on B220<sup>+</sup> cells. (C) Overlay histogram of the data in (B).



**Fig. 2.** Levels of various maturation markers on CD23<sup>-</sup> and CD23<sup>+</sup> transitional B splenocytes purified from post-irradiated mice compared with levels on B splenocytes from non-irradiated mice. The auto-reconstituted B splenocytes were stained with anti-CD23 and gated according to CD23 status.



**Fig. 3.** Expression of CD23 (A) and HSA (B) on purified B cells from the bone marrow and spleens before and various days after sublethal irradiation. Day 13–22 plots represent B cells from auto-reconstituting spleens. The bone marrow and mature B cells were purified from non-irradiated adult mice.

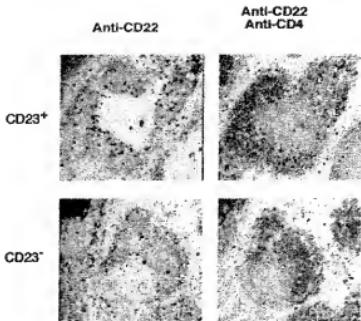


**Fig. 4.** (A) Susceptibility of CD23<sup>-</sup> and CD23<sup>+</sup> transitional B cells to anti-Ig-induced apoptosis. Purified B splenocytes from post-irradiated mice were stained with anti-BCR and anti-CD23, and sorted by flow cytometry into CD23<sup>-</sup> and CD23<sup>+</sup> transitional B cells. These two populations, along with B splenocytes from non-irradiated mice, were cultured for 16 h with indicated concentrations of rabbit anti-mouse Ig, then percent apoptotic cells (<2n) calculated by cell cycle analysis using propidium iodide as described in Methods. The data represent the mean of triplicates ± SD. (B) Proliferation of CD23<sup>-</sup> and CD23<sup>+</sup> transitional B cells to LPS and anti-Ig. CD23<sup>-</sup> and CD23<sup>+</sup> transitional B cells isolated as in (A), and B splenocytes from non-irradiated mice were treated with media, LPS (25 µg/ml) or rabbit anti-mouse Ig (50 µg/ml) for 64 h. Proliferation was quantitated by [<sup>3</sup>H]thymidine incorporation as described in Methods. The mean of triplicates ± SD is shown.

follicles where they can form germinal centers (20). In the absence of their specific antigen, the naïve B cells travel through the T zone and migrate to the B cell follicles.

After exiting the BM, newly formed B cells transit to the T cell-rich zones and red pulp of the spleen (21), but detailed data regarding the movement and localization of transitional immature B cells and their ability to enter B cell follicles has not been well characterized (22). We sorted auto-reconstituted transitional B cells by flow cytometry into CD23<sup>-</sup> and CD23<sup>+</sup>

subpopulations, and then labeled each population with the intracellular green fluorescent dye CFSE. These labeled transitional B cells were then separately injected into the tail veins of adult BALB/c mice. Approximately 16 h after transfer, the mice were sacrificed and their spleens were frozen and sectioned for staining. We observed that CFSE-labeled CD23<sup>-</sup> transitional B cells localized to the adult spleen and readily entered the B cell follicles during this time (Fig. 5). Flow cytometry on a part of the recipient spleen showed that the



**Fig. 5.** Immunohistochemistry photo-micrographs of contiguous splenic sections of non-irradiated adult mice injected 16 h previously with CFSE-labeled transitional B cells. Purified B splenocytes from post-irradiated mice were sorted by flow cytometry into CD23<sup>+</sup> and CD23<sup>-</sup> populations. After labeling with CFSE,  $5 \times 10^5$ – $10^7$  CD23<sup>+</sup> (top) or CD23<sup>-</sup> (bottom) transitional B cells were injected via the tail vein into recipient mice. (Left) The splenic sections were stained with anti-CD22 (red) and anti-FITC (dark blue). (Right) The sections were stained with a combination of anti-CD4 (red), anti-CD22 (blue) and anti-FITC (dark blue) which stains the CFSE-labeled cells.

transferred CD23<sup>+</sup> transitional B cells retained their HSA<sup>hi</sup> immature phenotype at the time of sacrifice (data not shown). In contrast, markedly fewer CFSE-labeled CD23<sup>-</sup> transitional B cells could be detected in the adult spleen during this time (Fig. 5). Owing to the small number of these cells, their maturity status and relative distribution within the spleen could not be determined with certainty.

#### CD23<sup>+</sup> and CD23<sup>-</sup> transitional B cells respond differently to T cell help signals

Although the two subpopulations of transitional B cells are susceptible to apoptosis and cell cycle arrest upon BCR ligation, the CD23<sup>+</sup> transitional B cells preferentially localized to the splenic follicles where they are in a position to interact with T cells. We therefore evaluated whether transitional B cells had the ability to respond to T cell-derived signals. We have previously shown that transitional B cells failed to undergo apoptosis could be rescued by the addition of IL-4 and/or activating anti-CD40 antibodies, mimicking both soluble and contact T cell activation signals (11). We added IL-4 and anti-CD40 alone or in combination after BCR ligation with anti-IgM F(ab')<sub>2</sub> and found that CD23<sup>+</sup> transitional B cells were rescued to a greater extent compared with CD23<sup>-</sup> transitional B cells from apoptosis. While CD40 stimulation had a greater impact than IL-4 to the survival of transitional B cells, the combination of the two signals appeared to play a synergistic role in their rescue. Similarly, CD23<sup>+</sup> transitional B cells proliferated to a greater degree to anti-IgM stimulation than their CD23<sup>-</sup> counterparts in the presence of the T cell signals (Fig. 6A and B).

Neither CD23<sup>+</sup> nor CD23<sup>-</sup> transitional B cells have the ability to up-regulate B7-2 to BCR-mediated stimulation

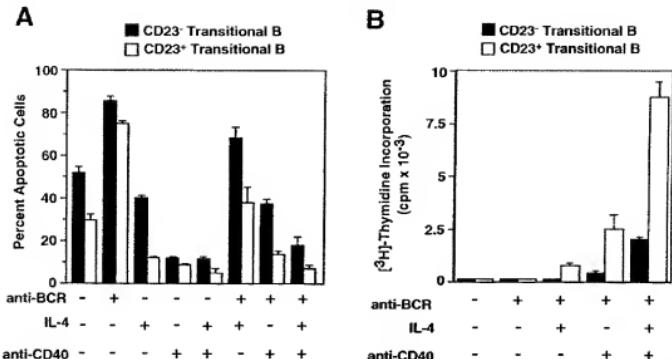
In order for complete activation of the T lymphocyte to occur, both the engagement of the TCR with MHC class II peptide and a second, antigen non-specific signal on the antigen-presenting cell (APC) is required (23). CD80 (B7-1) and CD86 (B7-2) are highly homologous molecules which are up-regulated in B cells in response to various activating stimuli including BCR ligation (24–27). Their up-regulation thus transforms a resting B cell into a fully competent APC (28).

We sought to examine the potential of transitional B cells for interacting with T cells. The interaction of TCR with the MHC class II:peptide and CD28 with B7 on mature B cells leads to the full activation of T cells with subsequent production of IL-2 and the induction of Bcl-x<sub>c</sub> (29). The inducible expression of B7 on immature B cells has been examined in cultured BM B cells from Ig transgenic mice (30). We sought to study the regulation of B7 in the heterogeneous subsets of transitional B cells isolated from the spleen. Although CD23<sup>+</sup> transitional B cells responded to T cell signals by being rescued from apoptosis and proliferating to BCR ligation, they failed to up-regulate B7-2 in response to BCR-mediated stimulation. Mature B cells increased their surface expression of B7-2 as early as 3 h after BCR cross-linking and maintained its expression for >24 h (Fig. 7). However, while expressing low levels of B7-2 on their surface constitutively, neither CD23<sup>+</sup> nor CD23<sup>-</sup> transitional B cells could be induced to up-regulate B7-2 in response to BCR ligation. Immature B cells failed to up-regulate B7-2 even 72 h after anti-Ig stimulation or after anti-Ig and CD40 co-ligation, and, as previously described (31), neither mature nor immature B cell subsets up-regulated B7-1 during the long incubation (data not shown). Apoptosis was prevented during the long incubation period with the caspase 3 inhibitor z-DEVD-FMK (32–34).

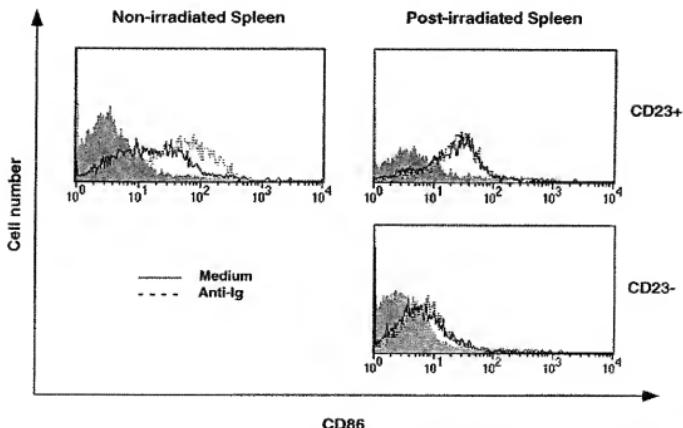
#### Discussion

Transitional B cells mark the critical link between immature B cells of the BM and fully immunocompetent mature B cells in the periphery. The ability of immature and transitional B cells to undergo negative selection in the bone marrow and in the periphery allows for the maintenance of tolerance to a variety of marrow and extra-marrow self antigens. Although all immature B cells share these characteristics, the variable expression of certain maturation markers such as B220, IgD, CD21, CD22 and CD23 within the immature B cell population suggested the existence of functionally distinct subsets of transitional B cells. We found that the HSA<sup>hi</sup> transitional B cells can be readily divided by CD23 expression into subsets corresponding to successive developmental stages and sought to better understand the functional significance of these subsets.

We found that CD23<sup>+</sup> and CD23<sup>-</sup> transitional B cells are similar in their susceptibility to apoptosis in response to BCR cross-linking, and proliferation profiles to various stimuli. CD23 status represents a continuum in the maturation of immature B cells and we found that CD23<sup>+</sup> transitional B cells localize to the splenic B cell follicles. Although CD23<sup>+</sup> transitional B cells were originally isolated from the spleen, transfer



**Fig. 6.** The degree of rescue from anti-Ig-induced apoptosis, and proliferation in CD23<sup>-</sup> and CD23<sup>+</sup> transitional B cells with the addition of T cell help signals. Transitional B cells purified from auto-reconstituting spleens were separated by FACS into CD23<sup>-</sup> and CD23<sup>+</sup> populations by staining with anti-B220 and anti-CD23. The two subpopulations were cultured in media alone, anti-Ig, IL-4 (1:20 dilution of X4 supernatant), anti-CD40 (1C10, 1  $\mu\text{g}/\text{ml}$ ) or combinations as indicated. (A) Percent apoptosis 16 h after culture was assessed using propidium iodide in cell cycle analysis. (B) Proliferation was assayed 64 h after culture using [ $^3\text{H}$ ]thymidine incorporation as described in Methods. The data shown represents the mean  $\pm$  SD of triplicates.



**Fig. 7.** Surface expression of CD86 after anti-Ig treatment in mature and transitional B cells. Purified B splenocytes from post-irradiated and non-irradiated mice were cultured in the presence of media alone or anti-Ig (50  $\mu\text{g}/\text{ml}$ ), and then stained with anti-CD86. The purified transitional B cell subpopulations were sorted by CD23 status. The purified B cells from non-irradiated mice were gated on the CD23<sup>+</sup> population. The shaded areas represent the isotype control.

experiments failed to show significant entry back into the spleen. However, without knowing their survival in the recipient mouse, it is difficult to make conclusions about their migration potential. Lastly, we found that responsiveness to T cell help is developmentally regulated and restricted to the CD23<sup>+</sup> transitional B cell subset.

Antigen encounter and productive interaction with T cells determine the fate of mature naïve B cells in the spleen (16,35,36), and recent studies implicate the follicular border as the area of cognate T-B cell interactions (37). We posit that B cell follicles are where CD23<sup>+</sup> transitional B cells, fated to undergo apoptosis upon antigen encounter, may be rescued in the presence of T cell help and perhaps be recruited into an ongoing immune response. We do not know whether intrinsic programs within the developing transitional B cell permit responsiveness to T cell signals at the CD23<sup>+</sup> stage or whether micro-environmental cues condition the early transitional B cell for responsiveness as it matures.

Developmentally regulated susceptibility to apoptosis may reduce the opportunity for self-reactive B cell clones to develop, but survival of late transitional B cells may be regulated by pre-existing activated T<sub>H</sub>1 cells. Rescue from clonal deletion may occur through bystander help, not requiring B7 and affecting those B cells reactive to endogenous antigens. Transitional B cells reactive to legitimate foreign antigens may be rescued by preactivated T cells in a cognate dependent interaction. This may be mediated by CD40 ligand without dependence on co-stimulation. For B cells expressing autoantigen reactive BCR, negative selection in the T cell compartment would prevent the cognate help they would need to be rescued. While attractive as a system to positively select useful clones from the developing B cells to expand the immune repertoire, this model could also render the host susceptible to the development of autoimmunity. If a self-reactive T cell were to escape negative selection in the thymus or if the antigen contains both self-B cell and foreign-T cell determinants, self-reactive B cells can escape negative selection (38,39). Ideally, the fact that developing T cells have a lower threshold for tolerance induction than B cells should ensure that the transitional B cells do not have self-reactive T cells that could rescue them (40). We found that CD40 had a greater effect than IL-4 on rescuing transitional B cells from BCR ligation-induced apoptosis and that both were required for full protection to occur. This implies that cell-cell cognate interactions may be more important for selection into the mature B cell pool than non-specific bystander effects of secreted cytokines.

Our finding that transitional B cells do not up-regulate the co-stimulatory molecule B7-2 in response to BCR cross-linking has implications for the outcome of T-B interactions on both the B and T cell compartments. Although resting B cells are poor APC for naïve T cells (and indeed may induce tolerance) (28,41,42), B cells activated through their antigen receptor or by other stimuli act as effective APC for T cells (through the up-regulation of co-stimulatory molecules (31,43–45)). In the case of antigen-activated transitional B cells encountering naïve T cells, absence of co-stimulation may lead to the induction of tolerance in the T cell compartment (28,46). This may act as a safeguard to silence those T cell clones reactive against soluble, extra-thymic self-antigens.

Recently, Greenwald *et al.* demonstrated that CTLA-4 is necessary for the induction of T cell tolerance, implying a necessity for the presence of B7 for tolerance to occur (47). In both mature and immature murine B cells, the constitutive level of B7-2, while reduced, is still present. After antigen encounter, the mature B cells up-regulate the level of surface B7-2, thereby making CD28:B7 the predominant interaction. In immature B cells, given the 20–50 times higher affinity of CTLA-4 compared with CD28 for B7 (48), the low, but not absent, levels of B7 will lead to a predominantly CTLA-4:B7 interaction.

We have better defined the subpopulation of developing B cells which possess the capacity to interact with T cells given their anatomic location and responsiveness to T cell help signals. It remains to study, in greater detail, the nature of the interaction between the transitional B cells and T<sub>H</sub>1 cells in the spleen, and the factors that dictate their possible outcomes, including B7. B cell subsets marked by the presence or absence of CD23 have also been described in mature B cells, and recent reports indicate that these subsets display differential responses to recombinant CD40 ligand and various T cell cytokines (49). Our studies utilize two of the signals known to play a role in the interaction of B and T cells. Future studies utilizing intact cognate T cells should provide a fuller picture of the spectrum of cytokines which play a role in the interaction between various transitional B cell subsets and T cells. The developmental regulation of antigen processing and presentation is an important related area meriting further study (50). Lastly, the molecular mechanisms involved in the maturation of transitional B cells and their selection into the long-lived pool are slowly being elucidated (12,51). This will contribute greatly to our understanding of the formation and maintenance of the normal immune repertoire as well as the development of autoimmunity.

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#### Abbreviations

APC	antigen-presenting cell
CFSE	carboxyfluorescein succinimidyl ester
BM	bone marrow
HSA	heat-stable antigen
LPS	lipopolysaccharide
sigM	surface IgM

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